

REMARKS

Status of the Claims

By this amendment, claims 1, 5-12, 16-17, and 22 are amended and new claim 53 is added. No new matter has been added. Upon entry of this Amendment, claims 1-24, 26-46, and 49-53 are pending. Claims 1-24 were restricted and examined. Claim 53 should be grouped with claims 1-24. Claims 26-46 and 49-52 have been withdrawn but should be rejoined with the examined claims as no art is cited of record.

General Comments

The Examiner is thanked for the helpful suggestions for amending language informalities.

Objection

The specification was amended to avoid the objection, which should be withdrawn.

Rejections- 35 U.S.C. § 112, first paragraph

Claims 8-13 and 22 are rejected under 35 U.S.C. § 112, first paragraph, on the basis that the subject matter was not described in the specification in such a way as to convey that the inventor had possession of the claimed invention. Applicants have amended claims 8-13 and 22 to comply with 35 U.S.C. § 112, first paragraph. Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection.

Rejections- 35 U.S.C. § 112, second paragraph

Claims 1-24 are rejected under 35 U.S.C. § 112, second paragraph, for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The rejection posits that claims 1 and 5 are vague in their recitation of the phrase "...in an opposite orientation...." In their amended form, claims 1 and 5 overcome these rejections. Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection.

Claim 6 is rejected on the grounds that it is unclear how the L1 and L2 sequences can be the same, when claim 1 recites the limitation that the L1 and L2 sequence cannot recombine with another. Applicants respectfully traverse and request reconsideration and withdrawal of the rejection.

Applicants submit that the specification, on page 9, lines 19-22, discloses "the recombinase specific to said SSRTS L1 and the recombinase specific to said SSRTS L2 are the same. By same recombinase it is meant that the recombinase specific to SSRTS L1 catalyzes recombination at SSRTS L1 and L2." Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection.

Claim 8 is rejected on the basis that it is vague and indefinite, Applicants have amended claim 8 to comply with 35 U.S.C. §112 and respectfully request withdrawal of the rejection.

The Office Action has rejected claim 10 on the grounds that it is unclear whether the claim language is open or closed. Accordingly, claim 10 has been amended to overcome this rejection. Applicants, therefore, respectfully request reconsideration and withdrawal of the the rejection.

Claims 9 and 12 are rejected on the basis that it is "unclear how different a protein can be from a recited protein and be considered a 'variant' of that protein." Applicants have amended claims 9 and 12 to overcome this rejection. Therefore, Applicants respectfully request reconsideration and withdrawal of the rejection.



Atty. Dkt. No. 065691/0219

Claims 16 and 17 are rejected on the basis that they recite a Markush group whose members include overlapping genuses. In their amended forms, claims 16 and 17 overcome these rejections. Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection.

CONCLUSION

As the above-presented amendments and remarks address and overcome all of the rejections presented by the Examiner, withdrawal of the rejections and allowance of the claims are respectfully requested.

If there are any questions concerning this application, the Examiner is courteously invited to contact the undersigned counsel.

Respectfully submitted,

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Date 3/3/3

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

The recombinase specific of said SSRTS is selected from the group of site—specific recombinases composed of the Cre recombinase of bacteriophage P1, the FLP recombinase of *Saccharomyces cerevisiae*, the R recombinase of *Zygosaccharomyces rouxii* pSR1, the A recombinase of *Kluyveromyces drosophilarius* pKD1, the A recombinase of *Kluyveromyces waltii* pKW1, the integrase λ Int, the recombinase of the GIN recombination system of the Mu phage, of the bacterial β recombinase or a variant thereof. In a preferred embodiment, the recombinase is the Cre recombinase of bacteriophage P1 (Abremski et. al., 1984), or its natural or synthetic variants.. Cre is available commercially (Novagen, Catalog No. 69247—1). Recombination mediated by Cre is freely reversible. Cre works in simple buffers with either magnesium or spermidine as a cofactor, as is well known in the art. The DNA substrates can be either linear or supercoiled. A number of mutant LoxP sites have been described (Hoess et al., 1986; Lee et al., 1998), indeed, the corresponding SSRTS L1 and/or L2 specific for said Cre recombinase are chosen from the group composed of the sequences Lox P1 (ATCC 53 254 et 20 773), Lox 66, Lox 71, Lox 511, Lox 512, Lox 514, Lox B, Lox L, Lox R and mutated sequences of Lox P1 site harboring at least one point mutation in the 8 nucleotide spacer sequence. In one embodiment, the point mutation is substitution of A for G at position 7 of the eight base spacer sequence of the wild type Lox P1 sequence, referred to herein as Lox511 sequence. Preferred SSRTS are Lox P1 (SEQ ID N^o NO. 1) and Lox 511 (SEQ ID N^o NO. 53)

Please replace the paragraph bridging pages 26-27 with the following paragraph.

Figure 2. Schematic representation of the construct pFIEXR and of the expected plasmids after Cre-mediated rearrangement. (A) pFIEXR (SEQ ID N^o NO. 54) contains, in the following order, the SV40 promoter (broken arrow), a loxP site (open arrowhead), a lox511 site (closed arrowhead), the coding sequence for the enhanced-green fluorescent protein (EGFP) linked to a poly-adenylation signal, the β -galactosidase promoter-less minigene (LacZ) in the antisense orientation, a loxP and a lox511 sites in inverted orientations. The SV40 promoter first drives the expression of EGFP. (B) Intermediate step after Cre-mediated inversion at the loxP sites. (C) Intermediate step after Cre-mediated

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inversion at the lox511 sites. **(D)** Final product after Cre-mediated excision between the two lox511 or the two loxP sites (asterisks). In this plasmid, SV40 promoter now drives beta-galactosidase expression. This reaction is not reversible, as the final plasmid contains single loxP and lox511 sites, which cannot recombine together.

Please replace the paragraph bridging pages 27-28 with the following paragraph.

Figure 3. *In vitro* Cre recombinase-mediated inversion/excision assay. (A)

Schematic drawing of the ploxLacZlox construct used to check for Cre preparation efficiency before (upper panel) and after (lower panel) Cre-mediated recombination. EcoRV restriction sites and location of probes 1 and 2 are indicated. **(B)** Schematic drawing of pFExR (SEQ ID N^o NO. 54) before (upper panel) and after (lower panel, pFExRrec) Cre-mediated recombination. EcoRV and XbaI restriction sites, together with location of probes 1 and 2 are indicated. **(C)** Evidence for Cre-mediated recombination by Southern blot analysis of plasmids digested with EcoRV and XbaI using probe 1. Lane 1 and 2, loxP-flanked LacZ plasmid (ploxLacZlox) ; lane 3 and 4, pFExR; lane 5 and 6, pFExRrec (inverted/excised pFExR, see Materials and Methods). A crude Cre preparation was added in reactions illustrated in lanes 2, 4 and 6, whereas a heat-inactivated Cre preparation was added in reactions shown in lanes 1, 3 and 5. **(D)** Evidence for Cre-mediated recombination probing the same Southern blot as in (C) using probe 2 (for details see Materials and Methods). Note that the excised lacZlox fragment (3.7kb) , which does not contain plasmid sequences, was lost during amplification in bacteria. Open arrowhead, loxP site; closed arrowhead, lox511 site.

Please replace the paragraph bridging pages 28-29 with the following paragraph.

Figure 5: Generation of a conditional RAR γ allele by homologous recombination.

(A) Schematic drawing of the RAR γ locus. Exons 7 to 14 are shown as solid boxes. As indicated, E7 is specific for RAR γ 2, while E8 to E14 are common to all isoforms. The promoter (P2) is indicated by a broken arrow. 5' and 3' untranslated regions are shown as white boxes. Exon 8, whose splice acceptor is shown as waved lines, was chosen for the conditional disruption of RAR γ . **(B)** Structure of the targeting vector (py6.0Flex β -Gal) (SEQ ID N^o NO. 55). **(C)** Structure of the recombinant allele following homologous

recombination. **(D)** Structure of the recombinant allele after FLP-mediated removal of the selection cassette.

Please replace the paragraph bridging pages 31-32 with the following paragraph.

Figure 9: *In vitro* Cre recombinase-mediated inversion/excision assay on plasmid pJMG (SEQ ID N^o NO. 56). **(A)** Schematic drawing of pJMG (upper panel), the intermediate construct pJMG-f (middle panel) and the final construct pJMG-fx (lower panel). HindIII restriction sites, together with the location of the probe are indicated. **(B)** Evidence for Cre-mediated recombination assessed by ethidium bromide stained agarose gel analysis of HindIII digested plasmids. Lane 1 and 2, loxP-flanked LacZ plasmid (ploxLacZlox); lane 3 and 4, pJMG; lane 5 and 6, pJMF-f (inverted pJMG, see Materials and Methods); lane 7 and 8, pJMG-fx (inverted and excised pJMG, see Materials and Methods). A Cre preparation was added in the reactions illustrated in lanes 2, 4, 6 and 8, whereas a heat-inactivated Cre preparation was added in the reactions shown in lanes 1, 3, 5 and 7. The sizes of the expected HindIII fragments are indicated on the right. **(C)** Evidence for Cre-mediated recombination assessed by Southern blot using a probe recognizing the rabbit beta-globin splice acceptor site (for details see Materials and Methods). Note that this probe does not hybridise to the ploxLacZlox. Open arrowhead, loxP site; closed arrowhead, lox511 site; closed flag, FRT site; open flag, FRTm site, SD, synthetic splice donor.

Please replace the paragraph bridging pages 35-36 with the following paragraph.

1.1. DNA Constructs. To construct plasmid pFIEXP (SEQ ID N^o NO. 54) (Fig. 1A), a loxP site, in the sense orientation, followed by a 21-bp spacer (oligos R1/R2; Table 1) was introduced into the EcoRI site of pSG5 (Green *et al.*, 1988). A lox511 site (Hoess *et al.*, 1986), also in the sense orientation, followed by a 21-bp spacer (oligos R3/R4) was introduced 3' to the loxP site. A second loxP site, in the antisense orientation, followed by a 21-bp spacer (oligos R5/R6) was introduced 3' to the first loxP and lox511 sites. A second lox511 site, also in the antisense orientation, followed by a 21-bp spacer (oligos R7/R8), was introduced 3' to the latter loxP site. The coding sequence of the enhanced green fluorescent protein (Zhang *et al.*, 1996) (EGFP; PCR-amplified using oligos R9/R10) and an NLS- β -galactosidase pA cassette (LacZ) (Bonnerot *et al.*, 1987) were introduced between the two sets of loxP sites, in the sense and the antisense orientation, respectively. Finally, the

remaining LacZ sequences of pSG5 were removed by digestion with BsaAI and SfiI, and repair by homologous recombination in *E. coli* using a SV40 promoter fragment (PCR amplified using oligos R11/R12). All cloning steps were checked by sequencing. The final constructs were again sequenced in all modified parts before starting *in-vitro* Cre-mediated recombination or cell culture experiments. Modifications were all carried out following standard protocols (Ausubel *et al.*, 1989). To obtain plasmid pFLEXRrec, pFLEXR was incubated with the Cre preparation (see below), and the recombined DNA was cloned in *E. coli*. pFLEXRrec structure was checked by restriction mapping and sequencing of the regions containing loxP and lox511 sites. Plasmids ploxlacZlox and pSG5-Cre have been described elsewhere (Feil *et al.*, 1997).

Please replace the table title and table on page 37 with the following table title and table. (Base pairs were underlined in the original text and not changed by this amendment.)

Table 1. Sequences of primers used for construction of the pFLEXR plasmid (SEQ ID N° NO 54).

Name	Sequence
R1	5' <u>==</u> ATTGATAACTTCGTATAGCATACATTATACGAAGTTATCCAAGCTTCAC CATCGACCCG-3' (SEQ ID N° <u>NO</u> 1)
R2	5' <u>==</u> AATTCGGGTCGATGGTGAAGCTTGGATAACTTCGTATAATGTATGCTAT ACGAAGTTATC-3' (SEQ ID N° <u>NO</u> 2)
R3	5'- AATTGCCAAGCATCACCATCGACCCATAACTTCGTATAGTATACATTAT ACGAAGTTATCG-3' (SEQ ID N° <u>NO</u> 3)
R4	5'- AATTCGATAACTTCGTATAATGTATACTATACGAAGTTATGGGTCGATG GTGATGCTTGGC-3' (SEQ ID N° <u>NO</u> 4)
R5	5'- CTAGTGGATCCGATAACTTCGTATAATGTATGCTATACGAAGTTATCCA AGCATCACCATCGACCCT-3' (SEQ ID N° <u>NO</u> 5)

R6 5'-
CTAGAGGGTCGATGGTGATGCTTGGATAACTTCGTATAGCATACATTAT
ACGAAGTTATCGGATCCA-3' (SEQ ID Nº NO. 6)

R7 5'-
CTAGTCCAGATCTCACCATCGACCCATAACTTCGTATAATGTATACTAT
ACGAAGTTATT-3' (SEQ ID Nº NO. 7)

R8 5'-
CTAGAATAACTTCGTATAGTATACATTATACGAAGTTATGGGTCGATGG
TGAGATCTGGA-3' (SEQ ID Nº NO. 8)

R9 5'-GGGGAATTCTTCTTGTACAGCTCGTCCA-3' (SEQ ID Nº NO. 9)

R10 5'-GGGGAATTCCCATGGTGAGCAAGGGCGAGGAG-3' (SEQ ID Nº NO. 10)

R11 5'-CTATCAGGGCGATGGCCCACTACGTGTTCTGAGGCGGAAAGAACCA-3'
(SEQ ID Nº NO. 11)

R12 5'-GGAATAGCTCAGAGGCCGAGGCGGCCTCGGCCTCTGCATAAATAAAA-
3' (SEQ ID Nº NO. 12)

Please replace the second paragraph on page 38 with the following paragraph.

1.2. *In vitro* Cre reactions. To perform Cre-mediated rearrangements *in vitro*, bacterial extracts containing an active Cre were prepared from *E. coli* 294-Cre strain 43. Cells were grown overnight at 37°C in 500ml LB medium, harvested by centrifugation, resuspended in 10ml Cre Buffer (50mM Tris/HCl pH 7.5, 33mM NaCl, 10mM MgCl₂, 5% glycerol, 0.02% NaN₃), and lysed by sonification. The soluble supernatant containing the Cre recombinase (Cre preparation) was recovered by centrifugation (14000 x g, 15min, 4°C). The relevant plasmids (3µg) were incubated with 100µl of the Cre preparation for 1 hour at 37°C. For the control reactions, Cre was heat-inactivated by incubating the Cre preparation for 10 min at 70°C. Plasmids were then isolated using the standard alkaline lysis method for DNA preparation (Ausubel *et al.*, 1989). The recovered DNA was then used to transform competent XL1-Blue cells, which were grown overnight in 2ml of LB at 37°C. Plasmids were isolated, digested by EcoRV and XbaI, separated on agarose gels and analyzed by Southern blotting using the radio-labelled oligos 5'-GTGCATCTGCCAGTTTGAGG-3' (SEQ ID Nº NO. 13) or 5'-AATACGACTCACTATAG-3' (SEQ ID Nº NO. 14) recognizing lacZ sequence or T7 promoter, respectively.

Please replace the paragraph bridging pages 39-40 with the following paragraph.

1.4. Construction of plasmid py6.0FIE β -Gal (SEQ ID N^o NO, 55). To construct plasmid py6.0FIE β -Gal, the RAR γ exon 8 splice acceptor (oligos G3/G4; Table 2) was inserted by homologous recombination in *E. coli* into an XbaI digested pBluescript SK+ (Pharmacia) containing a loxP site (oligos G1/G2) in the sense orientation at its NotI site and from which the LacZ sequences were removed. After insertion of a 62 bp fragment (oligos G5/G6) into the XbaI site, the (NLS) β -gal pA cassette (Bonnerot *et al.*, 1987) was introduced by homologous recombination in *E. coli*. A SnaBI and a lox511 site (oligos G7/G8) in the sense orientation was then introduced 5' of the loxP site into the SacII site. A second SnaBI site (oligo G9) was inserted into the BamHI site. An FRT site (oligos G10/G11) was inserted into the NotI site. The FRT/PGK/Neo/pA/FRT cassette was inserted into the XbaI site oligos G10/G11 giving rise to plasmid ploxP/lox511/lacZ/Neo. A loxP site was inserted in the sense orientation into the HpaI site of pSK γ 6.0 (Lohnes *et al.*, 1993) (oligos G12/G13). A lox511 site was introduced in the sense orientation into the 3' reconstructed HpaI site (oligos G14/G15). The EcoRI insert of this plasmid was ligated into a pGEX4T3 to obtain a LacZ sequence-deficient vector (pGEX γ 6.0-loxP-lox511). The SnaBI fragment from plasmid plox P-lox511-lac2-Neo was isolated and inserted into the SfiI site of pGEX γ 6.0/loxP/lox511 to obtain py6.0FIE β -Gal (SEQ ID N^o NO, 55).

Please replace the table bridging pages 40-41 with the following table. (Base pairs were underlined in the original text and not changed by this amendment.)

Table 2: Sequences of primers used for construction of the pγ6.0F1Exβ-Gal plasmid (SEQ ID N° NO. 55).

Name	Sequences
G1	5'-GGCCGCATAACTTCGTATAATGTATGCTATACGAAGTTAT- 3' (SEQ ID N° <u>NO.</u> 15)
G2	5'-GGCCATAACTTCGTATGCATACATTATACGAAGTTATGC-3' (SEQ ID N° <u>NO.</u> 16)
G3	5'-TATAATGTATGCTATACGAAGTTATTCCTTGGCCTGGAATTTGCAGA ATT-3' (SEQ ID N° <u>NO.</u> 17)
G4	5'-GCCCCGGGGGATCCACTAGT <u>TCTAGATGTCTCCACCGCTGAATGAAAA</u> GCA-3' (SEQ ID N° <u>NO.</u> 18)
G5	5'-CTAGTATGGATAAAGTTTTCCGGAATTCCGCT <u>TCTAGACTCATCAATGT</u> TATCTTATCATGTCTA-3' (SEQ ID N° <u>NO.</u> 19)
G6	5'-CTAGTAGACATGATAAGATAACATTGATGAGT <u>TCTAGAGCGGAATTCCG</u> GAAAACTTTATCCATA-3' (SEQ ID N° <u>NO.</u> 20)
G7	5'-GCT <u>ACGTAATAA</u> ACTTCGTATAATGTATACTATACGAAGTTATGGGTCG ATGGTGAGATCTCCGC-3' (SEQ ID N° <u>NO.</u> 21)
G8	5'-GGAGATCTCACCATCGACCCATAACTTCGTATAGTATACATTATACGA AGTTATT <u>ACGTAGCGC</u> -3' (SEQ ID N° <u>NO.</u> 22)
G9	5'-GATCTT <u>ACGTAA</u> -3' (SEQ ID N° <u>NO.</u> 23)
G10	3'-GGCCGGGAAGTTCCTATTCT <u>TCTAGAAAGTATAGGAACTTCCC</u> -3' (SEQ ID N° <u>NO.</u> 24)
G11	5'-GGCCGGGAAGTTCCTATACTTT <u>TCTAGAGAATAGGAACTTCCC</u> -3' (SEQ ID N° <u>NO.</u> 25)

G12 5'-AAGATAACTTCGTATAATGTATGCTATACGAAGTTATCCAAGCATCAC
CATCGACCCGTT-3' (SEQ ID Nº NO. 26) |

G13 5'-AACGGGTCGATGGTGATGCTTGGATAACTTCGTATAGCATACATTATA
CGAAGTTATCTT-3' (SEQ ID Nº NO. 27) |

G14 5'-AAGCCAAGCATCACCATCGACCCATAACTTCGTATAATGTATACT
ATACGAAGTTATGTT-3' (SEQ ID Nº NO. 28) |

G15 5'-AACATAACTTCGTATAGTATACATTATACGAAGTTATGGGTCGATGGT
GATGCTTGGCTT-3' (SEQ ID Nº NO. 29) |

Please replace the paragraph bridging pages 41-42 with the following paragraph.

1.5. Generation of the gene trap construct

To construct the plasmid pJMG (SEQ ID N° NO, 56), a PCR amplified PGK Neo cassette containing the OBS sequence and the synthetic splice donor site (SD; oligos J1/J2; Table 3) was introduced into the EcoRI site of pBluescript SK+ resulting in pJMG1. A cassette containing the FRT, loxP and lox511 sites was prepared by subsequent insertion of oligos J3 to J8 into a shuttle vector. This cassette was recovered by NruI and HindIII digest; repaired and introduced in front of the PGK-Neo gene of pJMG1. The lacZ sequence of the pBluescript SK+ was removed from pJMG1. A lox511 site (oligos J9/J10) and a FRTm site (oligos J11/J12) were subsequently introduced 3' to the synthetic splice donor site. The β -globin splice acceptor site (SA) followed by the IRES sequence were amplified by overlap extension PCR using oligos J13-J16. This fragment was introduced between the loxP site and the nls-LacZ polyA minigene of plasmid ploxP-nls-LacZ-pA. The obtained loxP-SD-IRES-nls-LacZ-pA DNA fragment was recovered and introduced, in antisense orientation at the BamHI site located in between the lox511 site and the PGK promoter to give to pJMG. The gene trap construct was excised from pJMG by NotI digestion and purification on a sucrose gradient.

Please replace the table bridging pages 43-44 with the following paragraph. (Base pairs were underlined in the original text and not changed by this amendment.)

Table 3: Sequences of primers used for construction of the plasmid pJMG.

Name	Sequences
J1	5'- ACTAGTGGATCCCCCGGGCTGCAGGAATTCTACCGGGTAGGGGAGGCGC TT-3' (SEQ ID N° <u>NO</u> , 30)
J2	5'-GTATCGATAAGCTTGATATCGCCGCTCGAGACTTACCTGACTGGCCGTC GTTTACAGTCAGAAGAACTCGTCAAGAAG-3' (SEQ ID N° <u>NO</u> , 31)
J3	5'-CTCGCGAGGAATTCAACCAGAAGTTCCTATTCTCTAGAAAGTATAGGAA CTTCCAGCT-3' (SEQ ID N° <u>NO</u> , 32)

- J4 5'-GGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCTGGTTGAATTCCTC
GCGAGAGCT-3' (SEQ ID Nº NO. 33)
- J5 5'-AATGCCTACCGGACCATCATAACTTCGTATAATGTATACTATACGAAGT
TATAAGCTTGCA-3' (SEQ ID Nº NO. 34)
- J6 5'-AGCTTATAACTTCGTATAGTATACATTATACGAAGTTATGATGGTCCGG
TAGGCATTTGCA-3' (SEQ ID Nº NO. 35)
- J7 5'-GAGCTCATAACTTCGTATAATGTATGCTATACGAAGTTATCCAAGCATC
ACCATATGCA-3' (SEQ ID Nº NO. 36)
- J8 5'-TATGGTGATGCTTGGATAACTTCGTATAGCATACATTATACGAAGTTAT
GAGCTCTGCA-3' (SEQ ID Nº NO. 37)
- J9 5'-TCGACATAACTTCGTATAATGTATACTATACGAAGTTATAC-3' (SEQ ID
Nº NO. 38)
- J10 5'-TCGAGTATAACTTCGTATAGTATACATTATACGAAGTTATG-3' (SEQ ID
Nº NO. 39)
- J11 5'-TCGAAGAAGTTCCTAATCTATTTGAAGTATAGGAACTTCGCGGCCGCA-
3' (SEQ ID Nº NO. 40)
- J12 5'-TCGATGCGGCCGCGGAAGTTCCTATACTTCAAATAGATTAGGAACTTCT-
3' (SEQ ID Nº NO. 41)
- J13 5'-CCGGTCCTTGGCCTGGAATTTGCACTCTGTTGACAACCATTGTCTCCT-3'
(SEQ ID Nº NO. 42)
- J14 5'-GTAATACGACTCACTATAGGGAATTCCGCCCCTCTCCCTC-3' (SEQ ID Nº
NO. 43)
- J15 5'-GAGGGAGAGGGGCGGAATTCCTATAGTGAGTCGTATTAC-3' (SEQ ID
Nº NO. 44)
- J16 5'-CTCCACCGCTGAATGAAAAGCAGCATGGTTGTGGCAAGCTTATCAT-3'
(SEQ ID Nº NO. 45)
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Please replace the paragraph on page 44 with the following paragraph.

1.6. *In vitro* Cre reaction for poly A trap experiments

To test for functionality of loxP and lox511 sites of pJMG, an *in vitro* Cre reaction was carried out (Schnütgen *et al.*, 2001). Briefly, a crude extract of *E. coli* 294-Cre cells (Cre preparation; Buchholz *et al.*, 1996) was incubated with 3 µg of the plasmids and the resulting DNA was transformed into *E. coli* DH5α and directly amplified in liquid medium. Amplified plasmid DNA was recovered and analysed by Southern blotting using the probe 5'-TAACAATTTACACAGGA-3' (SEQ ID N° NO, 46), recognising the rabbit β-globin intron splice acceptor sequence (Green *et al.*, 1988), to reveal the Cre-mediated rearranged constructs. To obtain the pJMG-f plasmid (Fig. 8B) pJMG was incubated for 5 min with the Cre preparation and transformed into *E. coli* DH5α. Individual clones were picked and analysed by restriction mapping and sequencing.

Please replace the paragraph on page 45 with the following paragraph.

1.8 RACE PCR

3' RACE was carried out as described by Frohman (1994). Briefly, a first RT-PCR was carried out using the oligo nucleotides Qt (5' -CCAGTGAGCAGAGTGACG AGGACTCGAGCTCAAGCT17-3') (SEQ ID N° NO, 47) as anchor primer, as well as Q0 (5'-CCAGTGAGCAGAGTGACG-3') (SEQ ID N° NO, 48) and Neol (3'-ACCGCTTCCTCGTGGTTTAC-3') (SEQ ID N° NO, 49) for amplification. An aliquot of 1 µl of this reaction was used for a nested amplification using Q1 (5'-GAGGACTCGAGCTCAAGC-3') (SEQ ID N° NO, 50) and Neo2 (5'-GCCTTCTTGACGAGTTCTTC-3') (SEQ ID N° NO, 51) primers. The resulting PCR fragments were purified using the NucleoSpin kit (Macherey-Nagel) and sequenced using the Neo2 or OBS (5'-CTGTAAAACGACGGCCAGTC-3') (SEQ ID N° NO, 52) primers.

Please replace the paragraph bridging pages 45-47 with the following paragraph.

EXAMPLE 1: *In vitro* site-specific recombination

The principle of the inventors' novel recombination strategy is illustrated in Figure 1. pFlExR (SEQ ID N° NO, 54), a pSG5-based reporter plasmid was designed (Figure 2) to test

its feasibility. It contains one pair of wild type loxP sites (open arrowheads), and one pair of lox511 sites (closed arrowheads), the loxP sites within each pair being oriented head to head. This organization (i.e. alternate loxP, lox511 and again loxP, lox511) is important. Both loxP and lox511 sites are recognized by Cre recombinase; however, they are “incompatible”, as lox511 sites can efficiently recombine with themselves, but not with loxP sites (Hoess *et al.*, 1986). Between the two sets of loxP-lox511 sites, the plasmid contains the coding region for the enhanced green-fluorescent protein (EGFP) in the sense orientation, and a promoter-less LacZ reporter gene in the antisense orientation. In this reporter plasmid, the SV40 promoter first directs expression of EGFP (Fig. 2A). Cre-mediated recombination may initially induce inversion of the intervening DNA at either the loxP sites (Fig. 23, open arrowheads), or the lox511 sites (Fig. 2B, closed arrowheads). Due to the reversibility of these reactions, an equilibrium between the states (A) and (B or C) is formed. However, inversion induces a direct repeat of either two lox511 sites (Fig. 2B; closed arrowheads) or two loxP sites (Fig. 2C; open arrowheads). A further Cre-mediated excision will then remove the DNA located between the two loxP or between the two lox511 sites (Fig. 2B and C; asterisks). In the resulting plasmid (pFlExRrec), single loxP and lox511 sites are left, making further inversion of the intervening DNA impossible (Fig. 2D). The SV40 promoter now drives expression of LacZ, instead of EGFP.

IN THE CLAIMS:

1. (Once Amended) An isolated DNA molecule comprising at least a sequence A flanked by at least a-site specific recombinase targeting sequences (SSRTS) L1, and at least a sequence B flanked by at least site specific recombinase targeting sequences (SSRTS) L2, said SSRTS L1 and SSRTS L2 being unable to recombine with one another, and wherein:

- (i) sequences L1 are in an ~~opposite-orientation~~ opposite to one another,
- (ii) sequences L2 are in an ~~opposite~~ orientation opposite to one another; and
- (iii) the order of SSRTS sequences in said DNA molecule is 5'-L1-L2-L1-L2-3'

5. (Twice Amended) The DNA molecule according to claims 1 to 4, wherein sequences A and B are in ~~an-a~~ direction opposite direction to one another.

6. (Twice Amended) The DNA molecule according to claim 1, wherein the site-specific recombinase sequence-specific of SSRTS-L1 and the site-specific recombinase targeting sequence and the recombinase specific of SSRTS L2 are the same.

7. (Twice Amended) The DNA molecule according to claim 1, wherein the said site specific recombinase targeting sequence (SSRTS) L1 and the site-specific recombinase targeting sequence-SSRTS-L2 are recognized by different site-specific recombinases.

8. (Twice Amended) The DNA molecule according to claim ~~6~~1, wherein the said site specific recombinase targeting sequence ~~are~~ is selected from the group of site-specific recombinases composed consisting of the Cre recombinase of bacteriophage P1, the FLP recombinase of *Saccharomyces cerevisiae*, the R recombinase of *Zygosaccharomyces rouxii* pSR1, the A recombinase of *Kluyveromyces drosophilarius* pKD1, the A recombinase of *Kluyveromyces waltii* pKW1, the integrase λ Int, the recombinase of the GIN recombination system of the Mu phage, ~~of the and~~ bacterial β recombinase or a variant thereof.

9. (Once Amended) The DNA molecule according to claim 8, wherein the said recombinase is the said-Cre recombinase of bacteriophage P1 ~~or its natural or synthetic variants~~.

10. (Once Amended) The DNA molecule according to claim 9, ~~characterized in that wherein~~ said SSRTSL1 and/or L2 specific for said Cre recombinase are chosen from the group consisting of Lox P1, Lox 66, Lox 71, Lox 511, Lox 512, Lox 514 and mutated Lox P1 sequences, wherein said mutated of Lox P1 sequences comprise site harboring at least one point mutation in the spacer sequence.

11. (Once Amended) The DNA molecule according to claim 10, wherein either SSRTS L1 comprises the Lox P1 nucleotide sequence (SEQ ID NO.^o 1) and SSRTS L2 comprises the Lox 511 nucleotide sequence (SEQ ID NO.^o 2) or SSRTS L1 comprises the Lox 511 sequence and SSRTS L2 comprises Lox P1 sequence.

12. (Once Amended) The DNA molecule according to claim 8, wherein the recombinase is the FLP recombinase of *Saccharomyces cerevisiae*, ~~or its natural or synthetic variants~~.

16. (Once Amended) The DNA molecule according to claim 15, wherein at least the sequences A and/or B are transcribed and translated, wherein said translated sequences coding code for at least one protein ~~selected in the group consisting of polypeptide, protein and protein fragments.~~

17. (Once Amended) The DNA molecule according to claim 16, wherein said protein is ~~selected in the group consisting of reporter protein, selection marker and a~~ protein of interest.

22. (Once Amended) The DNA molecule according to claim 21, wherein said autofluorescent protein is selected from the group consisting of the green fluorescent protein (GFP), the enhanced green fluorescent protein (EGFP), the red fluorescent protein (RFP), the blue fluorescent protein (BFP), and the yellow fluorescent protein (YFP) ~~and variants of these proteins.~~